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Level and Specificity of Antibodies Evoked by Crude and Purified Antigens of Poliovirus I and Echovirus 7

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ABSTRACT

FROMMHAGEN, LAURENCE H. (National Aeronautics and Space Administration, Ames Research Center, Moffett Field, Calif.). Level and specificity of antibodies evoked by crude and purified antigens of poliovirus I and echovirus 7. Appl. Microbiol. **13**:895–898. 1965.—Preparations of poliovirus I and echovirus 7, purified by density gradient centrifugation, liquid-phase partition, and anion exchange (diethylaminoethyl) chromatography, have been shown to evoke high antibody levels of substantial specificity in the complement-fixation assay. Certain practical aspects of the three purification methods are discussed. These results argue for the use of purified viral antigens, particularly in view of the simplicity of the purification methods now available.

It is the practice in many laboratories to use crude tissue culture antigens (tissue culture fluid clarified by centrifugation at 2,000 to 4,000 $\times$ *g* for 5 to 15 min) in the preparation of viral antisera. Since the viral antigen represents a small portion of the total protein of such tissue culture fluids, the principal antibody production is directed against the normal tissue culture antigens. In many cases where the spread between specific and nonspecific (tissue culture) antibody is slight, the antisera may, therefore, not be used in identification of viral isolates by the complement-fixation assay from the same or related tissue culture lines.

During the past several years, a number of techniques have been advanced for the purification of the enteroviruses. These include density gradient centrifugation (Frommhagen and Martins, 1961), anion-exchange chromatography (Hoyer et al., 1958), and liquid-phase partition systems (Albertsson, 1960). This report relates to the value of utilizing such purified preparations for the production of antisera, not only in terms of the potency and specificity of the antisera, but also according to the efficiency and convenience of the method.

MATERIALS AND METHODS

Preparation of enterovirus antigens. Poliovirus I (Mahoney) and echovirus 7 were grown in HeLa and monkey kidney cell monolayers as described elsewhere (Frommhagen and Martins, 1961). The chicken serum was omitted from the maintenance medium for reasons to be discussed. Prior to purification by density gradient centrifugation and diethylaminoethyl (DEAE) chromatography, the virus was concentrated 100- to 200-fold by centrifugation at 30,000 rev/min (70,000 $\times$ *g*) for 3 hr and suspension in 1% NaCl or the appropriate buffer.

Density gradient centrifugation. The concentrates of poliovirus and echovirus 7 viruses were centrifuged for 12 to 18 hr in a cesium chloride gradient with an initial density of 1.34. The infectious virus, which equilibrated in a narrow light-scattering band near the middle of the tube, was collected through a puncture in the bottom of the tube. The fraction was again centrifuged in cesium chloride in the same manner, and the infectious band was collected and dialyzed against 1% NaCl prior to ultraviolet-absorption analysis and inoculation into animals.

Liquid-phase partition. The unconcentrated tissue culture antigen of echovirus 7 was purified by alternate concentration into the bottom and top phases of a dextran sulfate-polyethylene glycol mixture according to the method of Albertsson (1960). The virus was removed from the top phase by centrifugation (70,000 $\times$ *g* for 3 hr) and was suspended in 1% NaCl.

DEAE chromatography. Column chromatography with the cellulose anion exchanger DEAE was applied to the purification of poliovirus I previously concentrated 100 to 400 times by ultracentrifugation (Hoyer et al., 1958). The fraction possessing the infectious virus was dialyzed against 1% NaCl.

Complement-fixation assay. A routine block-titration complement-fixation test (Schmidt and Lennette, 1955) was used in this study. Complement-fixation titers are reported as the limiting serum dilution required to fix two units of complement in a system containing tissue culture antigen diluted 1:4.

Neutralizing assay. The neutralizing potency of antisera was determined by the colorimetric metabolic inhibition test of Lipton and Steigman (1955). The neutralization titers are reported as the limiting serum dilution required to neutralize 100 to 300 TCD_{50} units of the virus.

Preparation of antisera. The crude tissue culture antigen or the purified virus suspended in 1% NaCl solution was administered intraperitoneally at weekly intervals to hamsters (three doses), guinea pigs (three doses), and monkeys (two doses). The volumes of the purified virus inocula were adjusted on the basis of the absorbance reading at 260 mμ so that they contained three to five times more viral antigen than was present in the crude tissue culture antigen. The preinoculation sera of all animals were shown to possess no detectable antibody to the antigen subsequently employed.

The ultraviolet-absorption properties of the purified viruses and normal HeLa cell material are summarized in Table 1.

TABLE 1. *Ultraviolet-absorption properties of purified virus suspensions and normal HeLa components*

Prepn	Method of purification	Principal absorption maximum	Optical density*
		mμ	
Poliovirus I	Density gradient	260	1.69
Poliovirus I	Liquid phase	276	3.4
Poliovirus I	DEAE chromatography	260	1.73
Echovirus 7	Density gradient	262	1.67
Normal HeLa material	—	278	3.0

* Ratio of optical density at 260 mμ versus optical density at 280 mμ.

RESULTS

The inoculation of crude tissue culture antigens into hamsters and guinea pigs resulted in the production of complement-fixing antibodies whose titers against infected tissue culture fluids were largely indistinguishable from those against normal tissue culture fluid (Tables 2 and 3). The neutralizing titers evoked to the purified antigen were higher than to the crude antigen, undoubtedly, in part, reflecting the greater amount of antigen in the purified antigen preparations.

The ultraviolet spectra of the purified virus preparations revealed that the density gradient and anion-exchange procedures yielded virus of a high degree of purity (Table 1). Although essentially all of the infectivity of poliovirus I was recovered from the liquid-phase system, the preparation was shown by ultraviolet analysis (Table 1) to contain large amounts of norma-

TABLE 2. *Antibody titers of antisera produced against poliovirus I and II, propagated in HeLa cell cultures, and purified by density gradient centrifugation in cesium chloride*

Animal	Antigen	Type	Neutralization titer[a]			CF titer[b] against poliovirus in TCF[c]			Normal TCF[d]
			Type I	Type II	Type III	Type I	Type II	Type III	
Hamster pool	Purified	I	16,346	<4	<4	2,048	<4	<4	16
Hamster pool	Crude	I	1,024	<4	<4	256	<4	<4	256
Guinea pig-1	Purified	I	1,024	<4	<4	512	<4	<4	4
Guinea pig-2	Purified	I	1,024	32	<4	512	<4	<4	4
Guinea pig-3	Purified	I	2,048	<4	<4	512	<4	<4	4
Guinea pig-4	Purified	I	1,024	<4	<4	256	<4	<4	4
Guinea pig-5	Crude	I	512	<4	<4	256	<4	<4	256
Guinea pig-6	Crude	I	256	<4	<4	256	<4	<4	256
Guinea pig-7	Crude	I	512	<4	<4	128	<4	<4	256
Guinea pig-8	Crude	I	512	<4	<4	512	<4	<4	256
Monkey-1	Purified	II	<4	16,384	32	64	4,096	64	32
Monkey-2	Crude	II	<4	16,384	<4	<4	1,024	<4	—

[a] Reciprocal of neutralization titer, expressed as the limiting serum dilution, determined by metabolic inhibition test.

[b] Reciprocal of complement-fixation (CF) titer, expressed as limiting serum dilution required to fix 2 units of complement.

[c] Reciprocal titer with tissue culture (monkey kidney) antigen diluted 1:4.

[d] Normal HeLa tissue culture fluid (TCF).

TABLE 3. *Complement-fixation titers of antisera (hamster) produced against echovirus 7 and polioviruses purified by density gradient centrifugation, liquid-phase partition, and DEAE chromatography*

Animal no.	Antigen	Type	CF titer*	
			Infected TCF†	Normal TCF‡
1	Echo 7	Crude	512	256
2	Echo 7	Crude	512	128
3	Echo 7	Crude	512	256
4	Echo 7	Density gradient	512	<8
5	Echo 7	Density gradient	512	16
6	Echo 7	Density gradient	256	<8
7	Polio 1	Liquid phase	2,048	64
8	Polio 1	Liquid phase	2,048	512
9	Polio 1	Liquid phase	1,024	64
10	Polio 1	DEAE chromatography	1,024	<8
11	Polio 1	DEAE chromatography	512	<8
12	Polio 1	DEAE chromatography	256	<8
13	Polio 1	DEAE chromatography	512	<8

* Reciprocal of complement-fixation (CF) titer, expressed as limiting serum dilution, required to fix 2 units of complement.

† Homologous tissue culture antigen diluted 1:4.

‡ Normal HeLa tissue culture fluid (TCF) diluted 1:4.

tissue culture components. It was, therefore, not surprising that the hamster antiserum prepared against poliovirus I, purified by the liquid-phase partition method, was markedly nonspecific (Table 3).

The slight complement-fixation titers to normal HeLa material in the antisera to poliovirus I and echovirus 7 (Tables 2 and 3), purified by density gradient centrifugation, relate to the results of another experiment. A quantity of normal HeLa tissue culture fluid was concentrated by centrifugation and fractionated in the same manner as the virus concentrates by density gradient centrifugation in cesium chloride. The fraction of the gradient corresponding to that at which the virus equilibrates evidenced some light-scattering and was shown, by ultraviolet-absorption analysis, to contain moderate amounts of normal HeLa cell material. A second cycle of density gradient centrifugation resulted in a smaller amount of this material in the middle fractions of the tube which, however, could not be reduced by a third density gradient cycle. For this reason, the viruses were submitted to two cycles of density gradient centrifugation. Virus preparations purified by only one cycle, when inoculated into hamsters, gave rise to highly nonspecific complement-fixation reactivities.

Preparations of poliovirus I, purified by the DEAE chromatographic method of Hoyer et al. (1958), evoked high levels of specific complement-fixation antibodies (Table 3); no antibodies were demonstrated, however, against the normal HeLa components. This finding correlates with the observation that corresponding eluates (0.02 M phosphate buffer) from DEAE chromatography of a concentrate of normal HeLa material was free from the host components, as determined by spectrophotometric analysis in the ultraviolet region.

The presence of serum in the maintenance medium brought about serious problems in subsequent purification by all three methods. Appreciable amounts of the serum proteins in the concentrates of tissue culture fluids led to irreducible amounts of these components in the virus fractions of the density gradients and to the appearance of these materials in the eluates from the DEAE columns containing the virus antigen. In addition, the presence of serum components markedly reduced the recovery of the infectivity of the virus (less than 20%) from the DEAE column. In the liquid-phase partition systems, voluminous amounts of precipitate appeared at the interface of the two phases when serum was present in the tissue culture fluid, making it impossible to separate suitably the two phases.

DISCUSSION

This investigation has demonstrated the advisability, if suitable and convenient methods are at hand, of purifying enterovirus antigens for the purpose of producing antisera for complement-fixation assay.

The density gradient method suffers from the disadvantages of any ultracentrifugal technique for routine use. The liquid-phase partition technique has not, in this laboratory, given virus preparations of very high purity. However, it does represent a very efficient and convenient concentration method, since all that is required is to add the proper amount of polyethylene glycol and dextran sulfate to a given volume of the tissue culture antigen and to permit the phases to form over 18 to 24 hr at 40 C.

The concentrate prepared by the latter procedure or by ultracentrifugation may then be purified by the simple method of DEAE chromatography described by Hoyer et al. (1958). It has been shown that this method yields the highest purity of virus, as evidenced in the specificity of the antibodies evoked against these preparations.

It is recommended that serum not be included during the maintenance of the monolayer cultures, since it has been found to interfere in the subsequent purification steps. This may be a disadvantage only in the case of an unadapted virus which requires that serum be present in order that the cell monolayer and the cells will remain intact long enough for the virus to attain maximal titer.

As shown in Tables 2 and 3, the antisera produced by inoculation of crude HeLa cell antigens did not react with the normal tissue antigens of monkey kidney cells. This simple solution to the problem of nonspecificity is inherent in most serological schemes, based upon the complement-fixation test, for the identification of the common viruses. However, some newly isolated, unidentified enteroviruses will replicate only in the same or a closely related kind of cells in which were grown the viral antigens used to produce the reference antiserum. In those circumstances which call for the identification of such isolates by the complement-fixation test, it is critical that there be available antiserum specific only to the viral antigen. Such antisera can only be obtained by the use of purified viral antigens.

The higher titers, particularly in the complement-fixation assays, engendered in many cases by the purified antigens appear to be due in large part to the larger amounts of the virus antigens in those preparations. These results are not amenable to any interpretation in regard to a possible "swamping" of antibody formation by the large amounts of host components and the consequent benefit of using purified antigen.

It can, however, be safely concluded that the virus does not lose any appreciable amount of its antigenicity by reason of purification. Nevertheless, the fact that the sera of a monkey and one guinea pig (see Table 2) reacted heterotypically indicates that some slight broadening of antigenicity, due perhaps to "denaturation," might occur occasionally during purification, or during storage of the purified antigen.

The low titers to normal HeLa components in some of the antisera against the purified preparations should not be interpreted as evidence for a "host antigen" in the enterovirus particle, since it was shown that small amounts of the normal tissue culture components were probably present in the purified preparations.

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